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During the past year of our grant we have concentrated on specific aim three. During the first year, we determined that NF2 blocks Ras transformation, in part, by blocking the action of the Rac small GTPase. Therefore, we focused our efforts on further assessing the mechanism by which NF2 antagonizes Rac signaling and function. Progress has been made in two main directions. First, we extended our previous analyses and determined that NF2 can block multiple Rac-mediated signaling activities. This suggests that NF2 inhibition of Rac function is not due to inhibition of a subset of Rac signaling events and argues that NF2 may directly modulate Rac itself. This outcome was an unexpected. Second, based on an observation by our collaborators, Rac was found to cause phosphorylation of NF2. The consequences of this phosphorylation on NF2 negative modulation of Rac transforming activity was determined. When taken together, these observations may provide a key clue regarding the tumor suppressing function of NF2. Rac function has been found to be necessary for the transforming actions of Ras and other oncoproteins (e.g., Mas, Vav, Abl, Fps). Therefore, if NF2 is a negative modulator of Rac function, it may serve as a negative modulator of growth regulation by a spectrum of growth promoting signaling proteins.

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Report for DAMD17-97-1-7342

Abstract

During the second year of our grant we have concentrated on studies described in Specific Aim 3. In our first year, we determined that NF2 blocks Ras transformation, in part, by blocking the action of the Rac small GTPase. Therefore, we emphasized studies to further evaluate the relationship between NF2 and Rac. During the course of these studies, we became aware of similar observations made by Drs. Tyler Jacks, Andrea McClatchey, and colleagues. Therefore, we have initiated collaborative studies with these investigators to evaluate NF2 interaction with Rac.

Introduction

Although the NF1 and NF2 candidate tumor suppressor genes have been implicated in the development of neurofibromatosis type 1 and 2, respectively, the precise functions of their encoded proteins remain to be elucidated. NF1 encodes neurofibromin, which has been shown to function as a GTPase activating protein (GAP) for the Ras proto-oncogene proteins. However, there is also evidence that neurofibromin may mediate downstream effector signaling pathways that promote Ras regulation of cellular differentiation. Whether neurofibromin is an effector of Ras remains to be determined. NF2 encodes merlin, a member of the ERM family of proteins that are believed to function in linking cytoskeletal components with membrane proteins. However, little is known concerning merlin function. Like neurofibromin, merlin expression has been shown to antagonize Ras transformation. However, how either protein blocks Ras transformation remains poorly understood.

Summary of Progress

Progress during the first year involved a determination that NF2 blocked Ras transformation and that this may be mediated by NF2 inhibition of Rac function. Consistent with this observation, NF2 inhibited Rac signaling functions. These observations provided to focus for our studies during the past year. The scope of these studies was also expanded to include collaborative studies with Drs. Andrea McClatchey and Tyler Jacks. Progress during the past year include: (1) a verification that non-epitope tagged versions of NF2 showed the same activity as what we observed previously with the epitope-tagged versions; (2) NF2 inhibition of multiple Rac-mediated signaling pathways; (3) NF2 inhibition of Rac-mediated growth transformation.

Body

Progress on Task C (specific aim three), to determine if merlin (NF2) inhibition of Ras is a consequence of antagonizing Ras via blocking Rho family protein function, was significant and will continue into the next year.

A. Structural characterization of NF2 antagonism of Rac signaling: role of N- and C-terminal sequences

Our results from the last year determined that NF2 could block Ras and Rac signaling. These studies were done using C-terminal epitope-tagged versions of full length NF2 as well as N-terminal and C-terminal fragments (provided by Nancy Ratner). However, in light of observations by other investigators that C-terminal manipulation can alter NF2 function, we felt that an important goal was to verify that our detected NF2 activities were not compromised by altered C-terminal function. Using non-epitope-tagged versions of full length (UM), N-terminal (UN), and C-terminal (UC) fragments of NF2, we confirmed our previous observations. Full length NF2 showed strong inhibition of Ras and Rac signaling (Jun activation) and the N-terminal fragment also showed a similar, but reduced, inhibition (Fig. 1). However, whereas our previous analyses found no inhibition by the C-terminus alone, we have seen low, but reproducible, inhibition of Rac signaling with the non-tagged C-terminal fragment. Thus, the inhibition seen with full length NF2 can be attributed primarily to sequences within the N-terminus and C-terminal sequences may contribute to this activity.

A surprising observation from the NF2 inhibition of signaling studies was that NF2 also blocked activated MEK1 stimulation of Elk-1 (see right panel in Fig. 1). MEK1 is not expected to activate Elk-1 in a Rac1-dependent fashion. However, since there are a number of observations that activated MEK1 can stimulate signaling pathways aside from direct activation of ERK (to then activate Elk-1), it may reflect the possibility that MEK1 activation of an autocrine pathway that then activates Elk-1 in a Rac1-dependent fashion has occurred. Elk-1 has been shown to be activated by Rac1 via MEK1 in published studies. The possibility that this represented a general growth inhibitory activity of NF2 was eliminated by our failure to see NF2 block the expression of a constitutively-activated luciferase gene expression vector. Also, we found no growth inhibitory activity with full length NF2 or either N- or C-terminal fragments, as assessed by the efficient isolation of stably-transfected NIH 3T3 cells that expressed high levels of each protein.

In addition to blocking Rac1 activation of Jun, we also found that co-expression of NF2 also blocked Rac1 activation of transcription from the cyclin D1 promoter as well as stimulation of serum response factor (SRF) activation. We showed previously, using Rac1 effector domain mutants, that Rac1 stimulates Jun, cyclin D1, and SRF via distinct effector signaling pathways. This general inhibition of Rac1 signaling suggested that NF2 may directly antagonize Rac1 function rather than blocking a signaling pathway downstream of Rac1.

B. NF2 antagonizes Rac transformation

We determined previously that NF2 blocked Ras focus-formation when assayed in NIH 3T3 cells and that NF2 could block Ras and Rac signaling. These results predicted that NF2 antagonism of Ras was due, in part, to blocking Rac function. To address this possibility, we determined if NF2 could antagonize Rac transforming activity. For these analyses we isolated NIH 3T3 cells stably cotransfected with a constitutively activated mutant of Rac1 (Q61L) and full length or truncated NF2. The isolated cell lines were first assessed to verify expression of NF2 using anti-NF2 antibody (provided by Dr. A. McClachey) Western blot analyses (data not shown). No difference in the morphology or the growth rate on plastic was observed for the

control Rac1(61L) and the co-transfected cell lines. However, we found that NIH 3T3 cells stably expressing activated Rac1 and NF2 were impaired in their ability to form colonies in soft agar.

Unpublished observations by Jacks and colleagues determined that activated Rac caused tyrosine phosphorylation of NF2. The significance of this phosphorylation event, with regards to NF2 antagonism of Rac, was then determined. The precise phosphorylation site was determined by Jacks and colleagues and this serine residue (S518) was mutated to either an alanine (518A; nonphosphorylatable) or to aspartate (518D; "constitutively phosphorylated"), We then determined the ability of these mutants to block Rac transformation.

For these analyses, NIH 3T3 cells were stably co-transfected with plasmid DNA expression vectors encoding activated Rac(61L), either with the empty pcDNA3 vector, or pcDNA3 encoding wild type (WT) or mutated (518A or 518D) NF2. Double drug selection was done to isolate transfected cell lines harboring both Rac and NF2, pooled together, and assessed for growth in soft agar (Fig. 2). Co-expression of wild type or 518A NF2 caused repression of Rac-induced colony formation in soft agar. In contrast, the putative constitutively phosphorylated version, 518D, actually showed enhancement of Rac-mediated transforming activity. These results suggests that Rac1-mediated phosphorylation of NF2 inhibits NF2 ability to block Rac function.

Future Directions

We will concentrate on elucidating the mechanism by which NF2 antagonizes Rac function. The role of Rac-mediated phosphorylation in modulating NF2 inhibition of transformation will be one goal of these studies. At present, it appears that NF2 may directly target all Rac function. How this is achieved is unclear and elucidating the details of this inhibition will be an important goal of our future studies. Additionally, since we and others have shown previously that Rac1 function is also necessary for the transforming actions of other oncoproteins, will NF2 also antagonize their transforming actions? For example, we showed previously that the Mas G protein-coupled receptor and that Vav guanine nucleotide exchange factor cause transformation in a Rac-dependent fashion. Therefore, we will determine if NF2 is also a modulator of Mas and Vav transforming activity. If so, is this also through inhibition of Rac1 function?

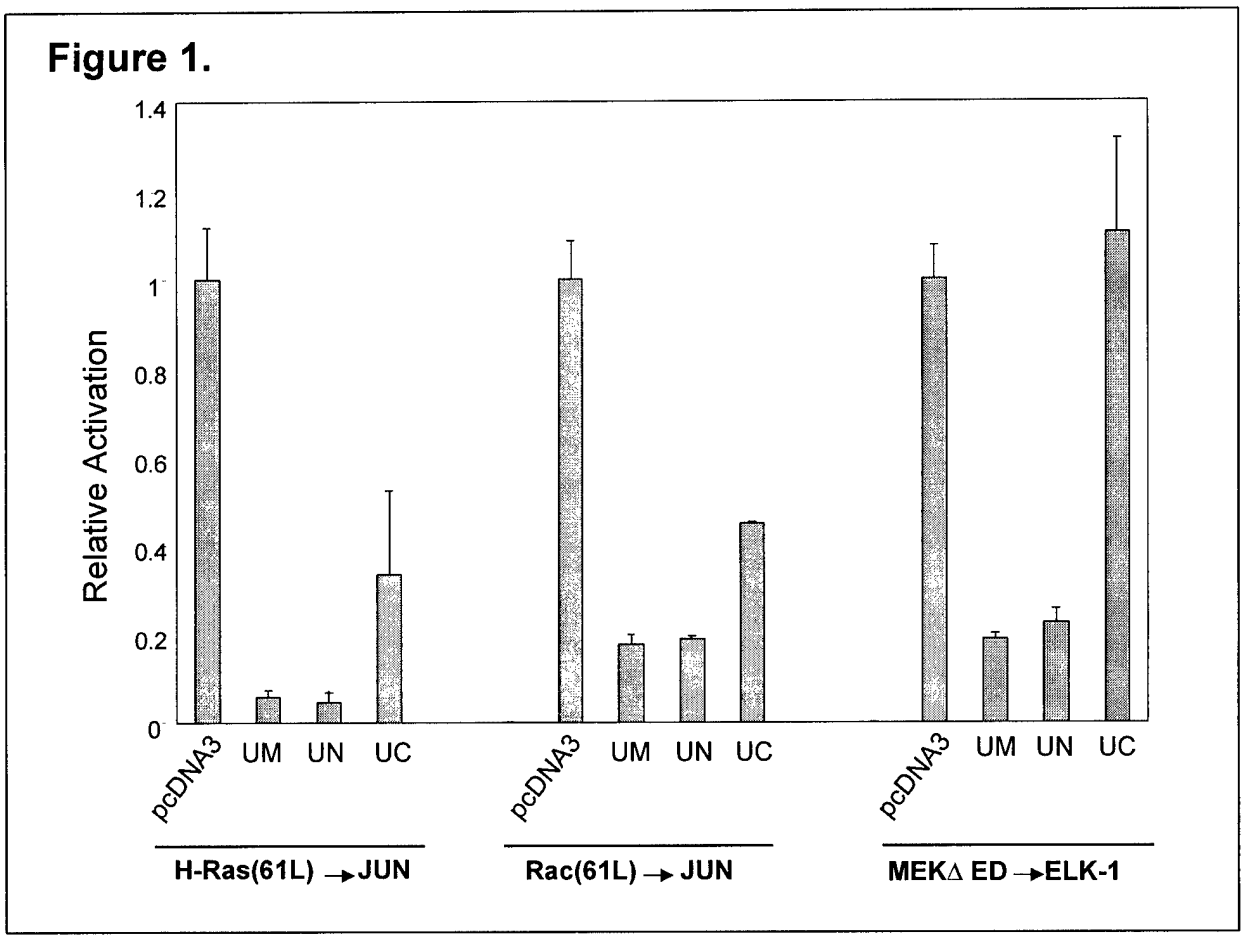

Figure 1. NF2 antagonizes H-Ras and Rac1 signaling. NIH3T3 cells were transiently cotransfected with the Gal-Jun or Gal-Elk and 5xGal reporter systems, constitutively activated H-Ras, Rac1 or MEK and NF2. Rac1 has been shown to be an activator of JNK mitogen-activated protein kinases, and JNK activates the Jun transcription factor. Cells were serum starved for 14 h in 0.5% serum and luciferase activity was measured the following day. Results represent the average of three independent experiments done in duplicate.

Transcriptional activation assays. NIH3T3 cells were transiently transfected via calcium phosphate precipitation with pcDNA 3 NF2 (0.7 μg), pCGN H-Ras(61L) (10 ng), pCGN-*rac*(61L) (0.5 μg), activated pcML MEKΔED (20 ng), 5X Gal-luciferase (2.5 μg) and Gal-Jun and Gal-Elk (0.5 μg) in 35 mm dishes. Cells were serum starved in 0.5% serum for 14 hr and luciferace activity measured on a luminometer. Relative light units (RLUs) are normalized to Rac(61L) plus empty pcDNA3 plasmid DNA.

Conclusions

Our results support a model where the tumor suppressing function of NF2 is mediated, in part, by its ability to antagonize Rac1 GTPase signaling and growth promotion. Rac1 is a regulator of cell cycle progression and cell proliferation. Rac1 also regulates actin organization, influencing

cell-cell and cell-substratum interactions. Rac1 also regulates gene expression via the activation of a spectrum of transcription factors, including serum response factor, c-Jun and NF-κB. Clearly, the ability of NF2 to antagonize Rac1 function may have significant consequences on the regulation of normal and neoplastic cell growth.

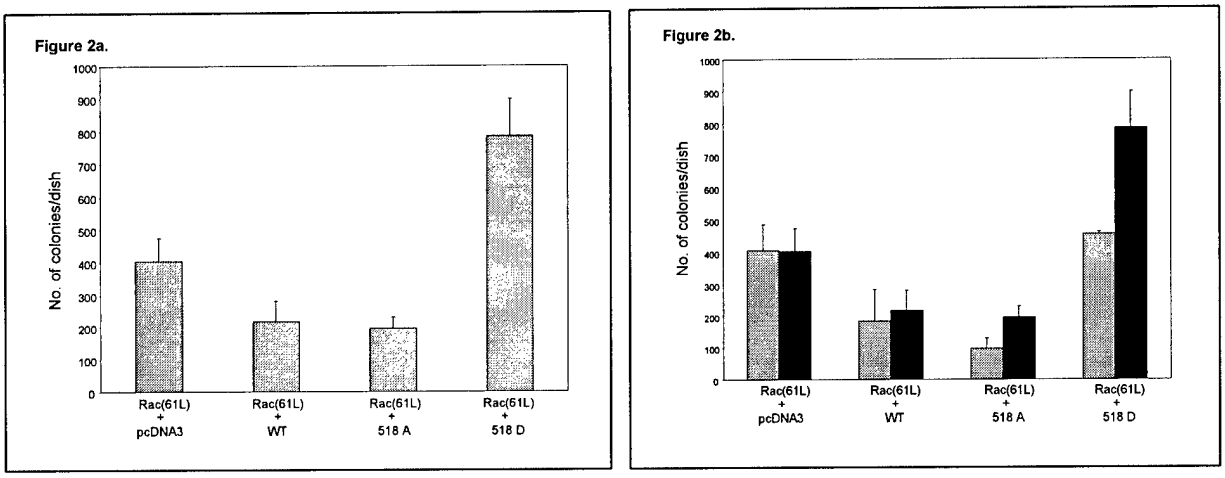

Figure 2. NF2 antagonizes Rac1-induced tumorigenic transformation. NIH 3T3 cells stably expressing constitutively activated Rac1 and NF2 were assayed for their ability to proliferate under anchorage-independent conditions. Cells were seeded in growth medium containing 0.3% agar and colonies were visualized after 14 days. Results represent (Panel A) the average of two experiments, (Panel B) data from two independent experiments.

Anchorage-independent growth assays. NIH3T3 cells transfected as above were co-selected in 0.4 mg/ml G418 (pcDNA3-NF2) and 0.1 mg/ml hygromycin B (pCGN-*rac*1(61L). Stably expressing cells were pooled and seeded at a density of 7.5×10^4 cells per 60 mm dish in DMEM containing 0.3% agar and 10% calf serum. Number of colonies was visualized after 14 days.

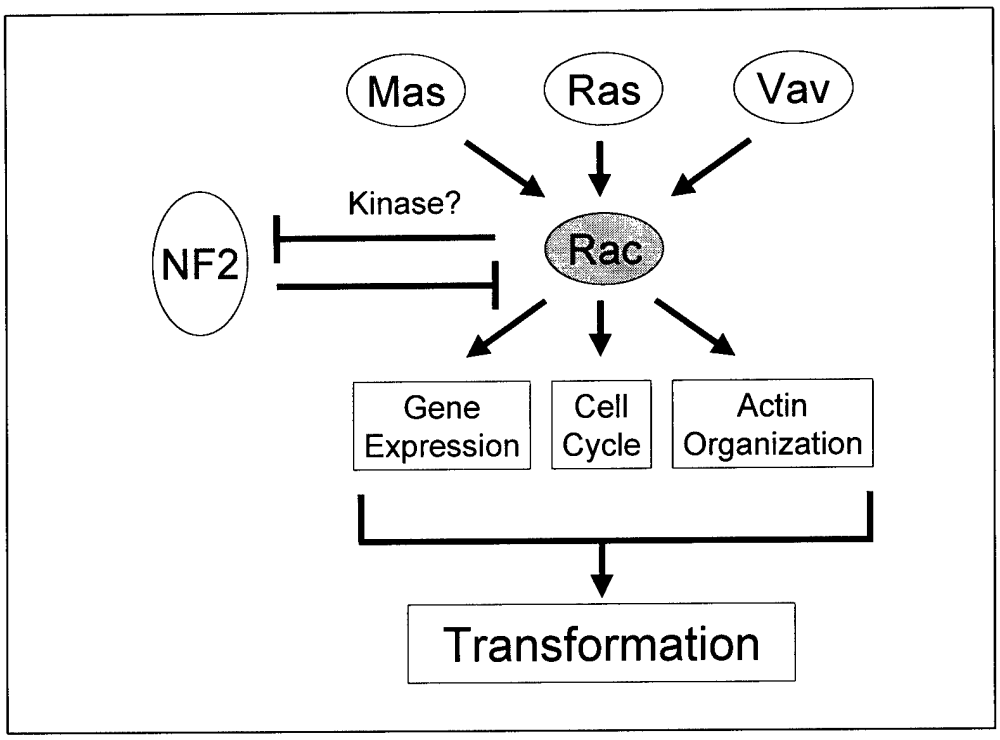

Figure 3. NF2 antagonism of Rac1- a general mechanism of growth inhibition by antagonizing multiple regulators of cell proliferation? We showed previously that Rac1 is required for Ras, Mas and Vav transforming activity. Mas is a novel G protein-coupled receptor that causing transformation by activation of Rac. Vav is a guanine nucleotide exchange factor that activates Rac1, as well as two related GTPases, Cdc42 and RhoA. We have shown that NF2 can antagonize Rac1 and Ras transforming activity. These observations argue that NF2 will also block the growth promoting actions of Mas and Vav. Whereas NF2 can block Rac function, Rac-mediated phosphorylation of NF2 may prevent NF2 inhibition of Rac function. Thus, we propose a model where NF2 and Rac1 can mutually antagonize each others functions, but Rac1 can escape NF2 inhibitory activity by causing its phosphorylation. In light of the diverse cellular functions of Rac1, involving the regulation of gene expression, cell cycle progression and growth, and actin cytoskeletal organization, the loss of NF2-mediated inhibition of Rac1 function can certainly contribute to aberrant Rac1-mediated oncogenesis.

Revised Statement of Work – In the review of the Annual Report (October 1998), a revised Statement of Work was requested. In light of the progress, as well as unexpected new directions, from our studies described in Task C, we will concentrate on expanding and completing these studies in Year 3. We feel that these studies have yielded the most fruitful and innovative observations of the three Tasks; therefore these studies will be of the highest priority for year 3. However, we also plan on a continuation of the work described in Tasks A and B, which were initiated during the first year, will continue to be pursued.

Task A	Continuation of these studies
Task B	Continuation of these studies
Task C	Continuation, as well as significant expansion, of these studies